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bcl-2–Immunoglobulin Transgenic Mice Demonstrate Extended B Cell Survival and Follicular Lymphoproliferation

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Summary

Human follicular B cell lymphomas possess a t(14;18) interchromosomal translocation that juxtaposes the putative proto-oncogene *bcl*-2 with the immunoglobulin (Ig) heavy chain locus. We generated minigene constructs representing the *bcl*-2–Ig fusion gene found at this chromosomal breakpoint. These constructs were placed into the germ line of mice to assess the effects of the t(14;18) during development. The transgene demonstrates a lymphoid pattern of expression and uniformly results in an expanded follicular center cell population. Hyperplastic splenic follicles coalesce to form massive regions of splenic white pulp. Mice over 15 weeks of age demonstrate regional lymphadenopathy with abnormal cellular infiltrates. The expanded lymphoid compartment is composed predominantly of polyclonal B220-positive, IgM/IgD-positive B cells. Provocatively, the *bcl*-2–Ig transgene confers a survival advantage to a population of mature B cells assessed in vitro. *bcl*-2–Ig transgenic mice document a prospective role for the t(14;18) in B cell growth and the pathogenesis of follicular lymphoma.

Introduction

Specific chromosomal translocations are found in distinct neoplasms but not their normal cellular counterparts, implicating them in the origin or maintenance of malignancy. The first interchromosomal translocation to be characterized at a molecular level juxtaposed a known cellular oncogene, c-*myc*, with the immunoglobulin (Ig) locus in Burkitt lymphoma and mouse plasmacytomas (Dalla-Favera et al., 1982; Taub et al., 1982; Sheng-Ong et al., 1982; Crews et al., 1982; Adams et al., 1983; Marcu et al., 1983). Transgenic mice possessing a *myc* gene fused to the Ig enhancer established the prospective importance of this translocation. Such Eμ–*myc* transgenic mice demonstrated a polyclonal pre-B cell hyperplasia (Langdon et al., 1986) that progressed over time to monoclonal malignancy (Adams et al., 1985; Leder et al., 1986). Moreover, transformation studies of human lymphoblastoid cell lines revealed that *myc* could complement the Epstein–Barr virus, providing experimental evidence for two major etiologic events in Burkitt lymphoma (Lombardi et al., 1987).

A second generation of chromosomal translocations found in lymphoid neoplasms introduce new putative proto-oncogenes into either the Ig loci (in B cell neoplasms) or T cell receptor loci (in T cell neoplasms) (for review see Korsmeyer, 1987). A prototype for this translocation is the t(14;18)(q32;q21) translocation found in 85% of follicular small cleaved B cell lymphomas and approximately 20% of diffuse lymphomas (Fukuhara et al., 1979; Yunis et al., 1987; Levine et al., 1985). The t(14;18) appears to occur early in pre-B cell development during attempted Ig gene rearrangement, introducing the *bcl*-2 gene from 18q21 into the Ig heavy chain locus at 14q32 (Tsujimoto et al., 1985; Bakhshi et al., 1985, 1987; Cleary and Sklar, 1985). This creates a *bcl*-2–Ig fusion gene at the derivative (der) 14 chromosomal breakpoint and results in a series of *bcl*-2–Ig chimeric RNAs (Cleary et al., 1986; Seto et al., 1988). However, the *bcl*-2 protein coding region is not interrupted but may display somatic mutation (Seto et al., 1988). The *bcl*-2 gene is normally expressed in pre-B cells, quiescent in resting B cells, expressed in proliferating B cells, but down-regulated in differentiated B cells (Graninger et al., 1987; Reed et al., 1987; Gurfinkel et al., 1987). In contrast, lymphomas with the t(14;18) display inappropriately elevated levels of *bcl*-2–Ig fusion RNA for their mature B cell stage of development (Graninger et al., 1987; Seto et al., 1988). The marked deregulation of the *bcl*-2–Ig fusion allele suggests it is the pathologically important consequence of the translocation.

The most comprehensive and unrestricted test of a gene's transforming capability is to place it transgenically into the germ line of mice. This provides a prospective opportunity to observe the effects of this gene on multiple cellular lineages during the development of an entire organism. To assess the contribution of the t(14;18) to neoplasia, we designed *bcl*-2–Ig minigenes representing the fusion gene found at the chromosomal breakpoint of human follicular lymphoma. The *bcl*-2–Ig transgene proved to be expressed within lymphoid tissue of all tested transgenic mouse lines. *bcl*-2–Ig transgenic mice uniformly develop an atypical lymphoid hyperplasia manifesting as markedly expanded splenic follicles that coalesce into massive geographic regions of white pulp. The deregulated *bcl*-2–Ig transgene prolongs the survival of a polyclonal population of mature B cells responsible for this expanded compartment of predominantly follicular center cells. These transgenic mice document a prospective role for the t(14;18) and its deregulated *bcl*-2–Ig fusion gene in the pathogenesis of B lymphoid neoplasms.

Results

bcl-2–Ig Minigene Constructs

We reconstructed the molecular consequence of the t(14;18)(q32;q21) by generating minigene constructs of the *bcl*-2–Ig fusion gene found on the der (14) chromosome (Figure 1). The enormous size of *bcl*-2 intron II (~350 kb)

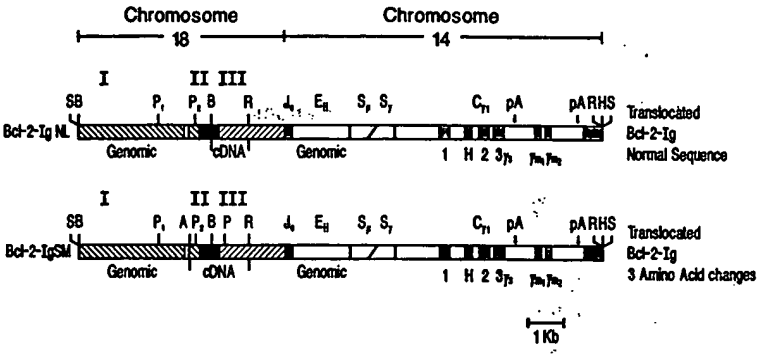


Figure 1. The 17.5 kb *bcl*-2–Ig Minigene Fragments Used for Microinjection

bcl-2–Ig NL possesses the normal germ line sequence for the 239 amino acid *bcl*-2 protein (filled box), while *bcl*-2–Ig SM encodes a three amino acid difference found in the SU-DHL-6 t(14;18) cell line, perhaps reflecting somatic mutation. These constructs possess a central region of cDNA that contains the exon II–exon III juncture, eliminating the ~350 kb intron II. *bcl*-2 intron I and numerous introns within the Ig heavy chain gene are present (open boxes). The germ line genomic clone of *bcl*-2 provides both promoters (P_1 and P_2) and 5′ flanking sequence. A genomic clone of the der (14) breakpoint of SU-DHL-6 provides the juncture between the MBR of *bcl*-2 and the joining (J6) region of the Ig heavy chain locus, including the enhancer (E_H) switch S_μ/S_γ and constant ($C_{\gamma 1}$) regions. Restriction sites: BamHI (B), PstI (P), EcoRI (R), AccI (A), HindIII (H), SalI (S). See Experimental Procedures for details of construction.

precluded the use of the entire gene and prompted the inclusion of a small portion of cDNA containing the exon II–exon III juncture. Two forms of the minigene were generated by varying the extent of cDNA used. *bcl*-2–Ig NL possesses a normal *bcl*-2 protein coding sequence, whereas *bcl*-2–Ig SM encodes the three amino acid changes present in the human B cell lymphoma line SU-DHL-6, which bears the t(14;18). *bcl*-2–Ig SM was created in the event that this potential somatic mutation was necessary for transforming function. Both constructs introduce the Ig heavy chain gene including the joining, enhancer, and constant γ1 regions at the *bcl*-2 major breakpoint region (MBR), as typically occurs in t(14;18) lymphomas (Figure 1).

bcl-2–Ig Transgenic Mice

bcl-2–Ig NL and *bcl*-2–Ig SM minigenes were microinjected into the pronuclei of C57BL/6 × C3H/He F1 fertilized eggs, and two-cell stage embryos were reimplanted into pseudopregnant ICR outbred females. DNA from tail biopsies identified nine founder animals and determined their pattern of integration (Table 1). Eight founders possessed the *bcl*-2–Ig NL construct, and one founder, M-23, possessed the *bcl*-2–Ig SM form. We initiated long-term lines by breeding founder mice with F1 mice and screening tail biopsies of offspring. Five *bcl*-2–Ig NL lines and one *bcl*-2–Ig SM long-term line have been established to date (Table 1).

Tissue-Specific Expression of *bcl*-2–Ig Minigenes

The tissue-specific expression of the *bcl*-2–Ig transgene was examined within a variety of organs from available animals of established lines (Figure 2 and Table 1). A discriminatory S1 nuclease protection assay recognized transcripts originating from the transgene protecting a 401 nucleotide fragment. It did not hybridize with the endogenous murine *bcl*-2 RNA in tissues or RNA from the highly expressing murine pre-B cell line 70Z/3 (Figure 2). All transgenic mice examined demonstrated a lymphoid pattern of expression, revealing transcripts in spleen and also thymus when tested between 8 and 13 weeks of age. Overall, the level of *bcl*-2–Ig RNA was somewhat less than in

Table 1. *bcl*-2–Ig Transgenic Founders and Lines

Founder Number	Construct	Offspring (transgene⁺/total)	Long-Term Line	Transgene Expression						
				Brain	Thymus	Heart	Lung	Spleen	Liver	Kidney
23	*bcl*-2–Ig SM	39/85	+	–	+	–	–	+	–	–
9	*bcl*-2–Ig NL	0/26	–	ND	ND	ND	ND	ND	ND	ND
14	*bcl*-2–Ig NL	2/13	+	ND	ND	ND	ND	ND	ND	ND
30	*bcl*-2–Ig NL	13/20	+	–	+	–	–	+	–	–
37	*bcl*-2–Ig NL	0/11	–	ND	ND	ND	ND	ND	ND	ND
43	*bcl*-2–Ig NL	50/80	+	–	+	–	+	+	–	–
57	*bcl*-2–Ig NL	3/4	–	–	ND	–	ND	+	–	–
59	*bcl*-2–Ig NL	1/6	+	ND	ND	ND	ND	ND	ND	ND
62	*bcl*-2–Ig NL	32/60	+	ND	+	–	–	+	–	–

Transgene expression was determined by S1 nuclease protestion assay (see Experimental Procedures).
ND, not determined.

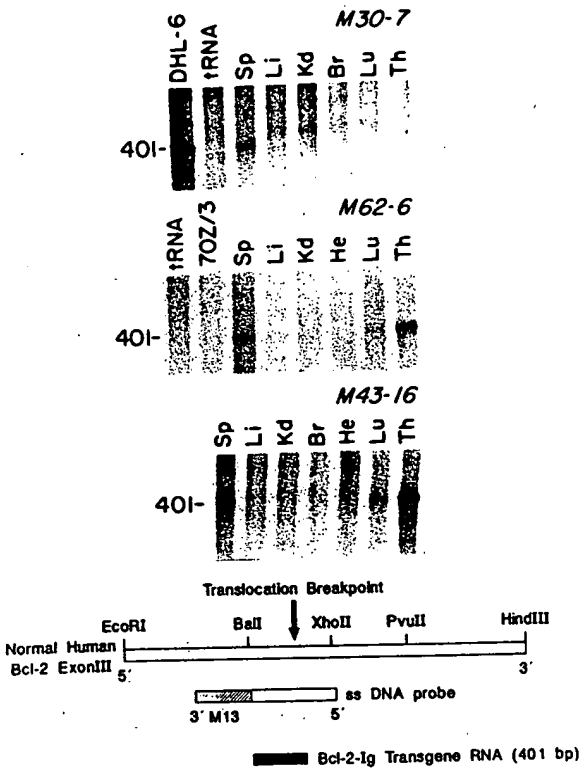


Figure 2. S1 Nuclease Protection Assay Using a Reverse Complementary Single-Stranded DNA Probe Possessing the *bcl*-2 Genomic MBR and Additional M13 Phage Sequences

Transcripts from either *bcl*-2–Ig transgene protect the same 401 nucleotide DNA fragment as transcripts from line SU-DHL-6. RNA (20 μg) from transgenic spleen (Sp), liver (Li), kidney (Kd), brain (Br), lung (Lu), heart (He), and thymus (Th) were hybridized with the radiolabeled probe. M-30-7 was 10 weeks of age, M-62-6 was 13 weeks, and M-43-16 was 8 weeks.

the SU-DHL-6 cell line. The M-43 transgenic line also displayed expression of *bcl*-2–Ig NL in the lung, perhaps reflecting the influence of the integration site.

bcl-2–Ig Transgenic Mice Develop Polyclonal Lymphoid Hyperplasia

Modest splenomegaly was noted in *bcl*-2–Ig transgenic mice when sacrificed after 20 weeks of age. Light microscopic appearances of spleens from transgenic mice at 8–25 weeks of age varied markedly from their normal littermates for all lines (M-23, M-30, M-43, M-62) examined. Extensive hyperplasia of white pulp zones was observed. Contiguous nodules frequently coalesced into large geographic regions of white pulp (Figure 3A). These areas were irregular in outline, yet the mantle zone was preserved and no red pulp invasion was seen. Expanded follicular center areas possessed predominantly small follicular center cells and occasional immunoblasts, macrophages, and plasma cells. Mitotic figures were rarely observed. A peripheral cortical zone composed of small mature lymphocytes was present. Germinal centers were scattered eccentrically in the white pulp zones (Figure 3A).

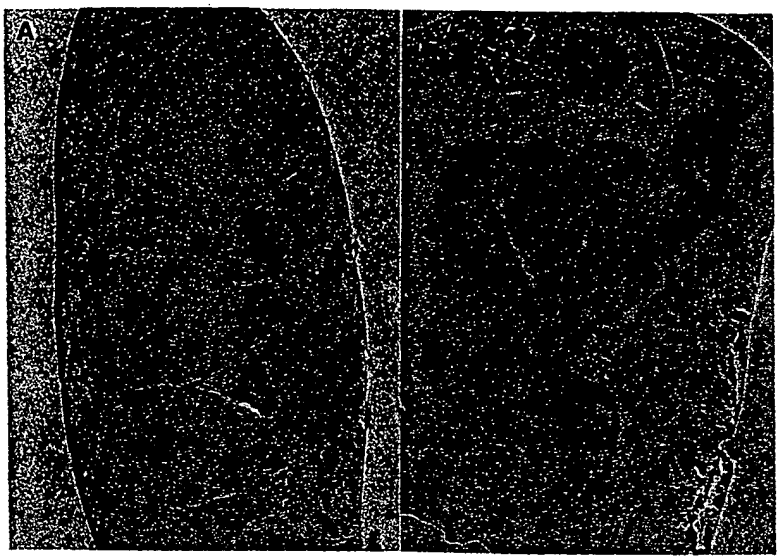
Immunofluorescence staining of transgenic spleens indicated that the expanded white pulp zones were composed predominantly of B lymphocytes expressing surface IgM, IgD, and K (Figure 3B). The periarterial white pulp region was composed of T cells expressing Thy-1, L3T4 (CD4), and Lyt-1 (CD5) (Figure 3B). Whereas the splenic follicles in transgenic mice were greatly enlarged, the topographic distribution of B and T cells within the white pulp was relatively normal.

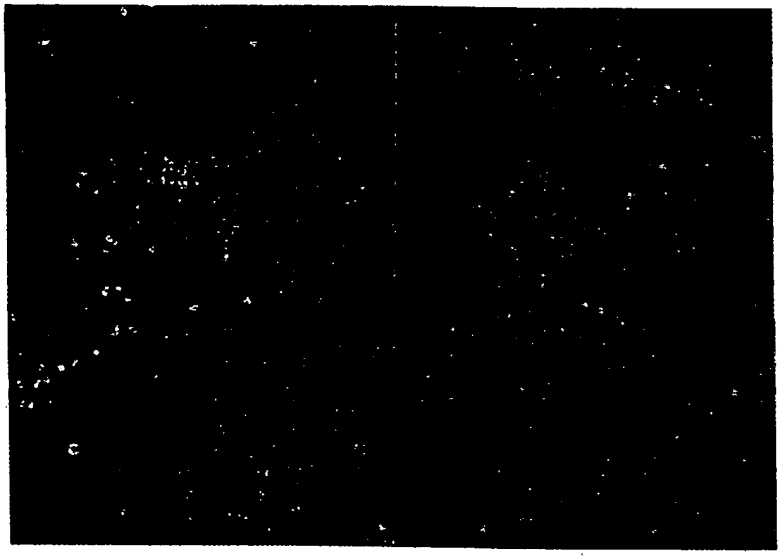
Mesenteric and para-aortic lymph nodes from M-23, M-30, and M-62 lines older than 15 weeks of age displayed 2- to 3-fold enlargement. However, adenopathy of other regional and peripheral lymph nodes has not been observed in animals of up to 25 weeks of age. Cortical zones displayed lymphoid follicles and scattered germinal centers (Figure 3C). Medullary cords and interfollicular areas at times were abnormal, with expansion by infiltrates of mixed small and large cleaved follicular center cells, immunoblasts, and numerous plasma cells (Figure 3C). These infiltrates were not observed in lymph nodes from corresponding normal littermates.

Despite expansion of splenic white pulp and the abnormal composition of lymph nodes, there was no evidence of monoclonality. Analysis of the endogenous Ig heavy chain gene configuration revealed no clonally rearranged population above the 5% threshold in spleens or lymph nodes of transgenic mice up to 25 weeks of age (Figure 4). Genomic blots also revealed no β T cell receptor rearrangements (data not shown). No consistent changes in brain, heart, lung, liver, kidney, gonads, or gastrointestinal tract were noted on tissue sections stained with hematoxylin and eosin (data not shown).

Expanded B Cell Compartment in *bcl*-2–Ig Transgenic Mice

The total splenic mononuclear cells were modestly increased in transgenic mice of 8–19 weeks of age. The spleens of these mice possessed an average of $146 \times 10^6 \pm 76 \times 10^6$ ($n = 21$) total mononuclear cells, compared with normal littermates that possessed $88 \times 10^6 \pm 35 \times 10^6$ ($n = 15$). The precise immunophenotype and quantitation of the expanded lymphoid compartment was addressed by performing flow cytometry analysis on spleen cells from lines M-23, M-43, and M-62. Contour plots of splenic cells analyzed by flow cytometry from a 9-week-old representative transgenic, M-62-55$^+$, are contrasted with a normal littermate, M-62-56$^-$, in Figure 5. An expansion of relatively normal-sized B cells was observed. The percentage of cells positive for B cell markers was increased in the transgenic mouse (B220, 61% vs. 25%; Ia, 64% vs. 37%; IgD, 53% vs. 18%; K, 54% vs. 33%; AA4.1, 44% vs. 19%) (Figure 5). The absolute number of B cells was increased in the spleens of transgenics versus normals; for example, B220-positive cells were 6-fold greater in M-62-55$^+$ versus M-62-56$^-$. The AA4.1 marker is found commonly on pre-B cells and their progenitors (McKearn et al., 1984; McKearn and Rosenberg, 1985), while the other markers indicate an expansion of mature B cells (positive for B220, Ia, IgM, IgD, K). The Lyt-1–positive cell population was not expanded, indicating





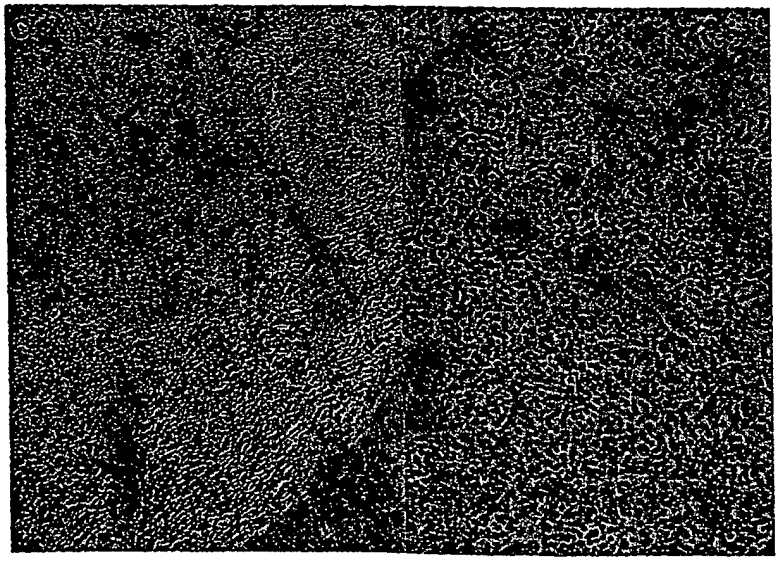


Figure 3. Histology of Normal and Transgenic Spleen and Lymph Node

(A) Sections of spleen from a normal littermate (left, 50×) and a 12-week-old M-23 transgenic line (right, 42×), stained with hematoxylin and eosin.

(B) Immunofluorescence photomicrographs of a frozen tissue section (5 μm) of spleen from an 8-week-old M-23 transgenic mouse. Anti-mouse IgM immunofluorescence is displayed on the left (60×), anti-L3T4 immunofluorescence on the right (60×).

(C) Section of para-aortic lymph node from a 25-week-old M-23 transgenic mouse, stained with hematoxylin and eosin. The paracortical zone and medullary cords (left, 100×) are expanded by a mixed lymphocytic infiltrate of follicular center type cells, immunoblasts, and numerous plasma cells (right, 240×).

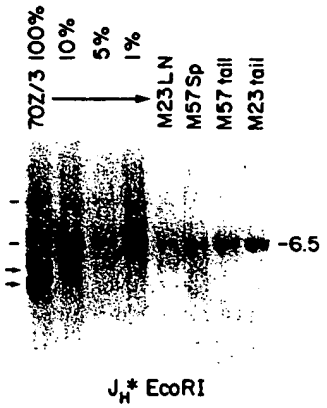


Figure 4. Genomic Blots of 10 μg of EcoRI-Digested DNA Probed with a Murine 1.0 kb XbaI–EcoRI Ig J_H Region Probe

The 70Z/3 murine pre-B cell line is diluted with liver DNA. The germ line EcoRI J_H-containing fragment is 6.5 kb. DNAs from lymph node (LN), spleen (Sp), and tail are presented for transgenic lines M-23 (16 weeks of age) and M-57 (13 weeks of age).

that the B cells were not of the CD5 subset. The percentage of T cells was proportionately decreased in the spleens of transgenics as represented by Thy-1 (16% vs. 33%) in Figure 5; however, the absolute number of splenic T cells was roughly comparable in transgenic and normal mice. Flow cytometry of bone marrow and lymph node cells also revealed a B cell expansion (data not shown).

bcl-2–Ig Transgenic Mice Display Hypergammaglobulinemia

The presence of immunoblasts and plasma cells within the spleen and lymph nodes suggested that B cells bearing the *bcl*-2–Ig transgene were capable of differentiation. Further evidence that the transgene does not block maturation or heavy chain class switching was provided by determining serum immunoglobulin levels. Approximately 2-fold elevations in IgG and IgA were observed, while the IgM levels in transgenic mice were comparable to those in normal littermates (Figure 6).

The *bcl*-2–Ig Transgene Promotes Survival of B Cells In Vitro

An unexpected observation was made when single-cell suspensions of splenocytes were placed in culture in DMEM supplemented only with 5% fetal calf serum. The vast majority of splenocytes from normal littermates died within 4 days in vitro, and nearly all lymphocytes were dead by 14 days (Figure 7A). Cultures from transgenic animals (M-23, M-30, M-43, M-62) demonstrated a slower,

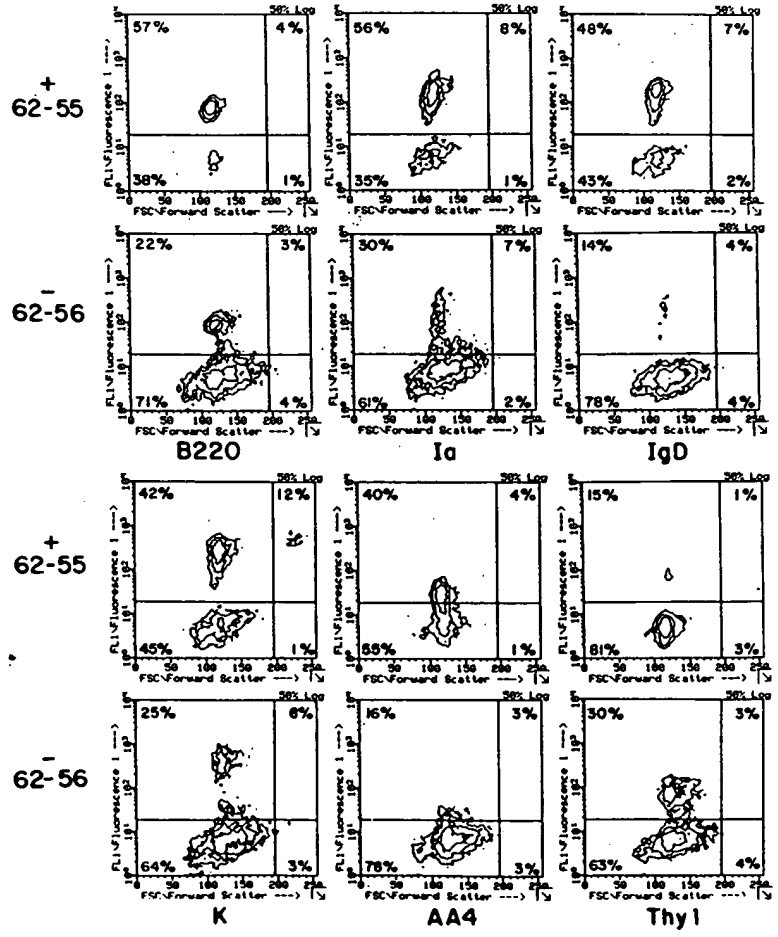


Figure 5. Contour Plots of Viable Splenic Mononuclear Cells Stained by Indirect Immunofluorescence and Analyzed by Flow Cytometry

bcl-2–Ig transgenic M-62-55$^+$ was compared with a normal littermate, M-62-56$^-$. Dead cells were discriminated by differential uptake of propidium iodide and were excluded from analysis by gating. The horizontal cursor line was positioned based on cells stained with second antibody alone. Using this cutoff, the precentage of positive cells with second antibody alone varied between 2% and 8%. The vertical cursor line was arbitrarily placed on the basis of the size range of normal cells from M-62-56$^-$. Contour levels were plotted with a threshold level of 10%. The percentage of cells in each quadrant is noted.

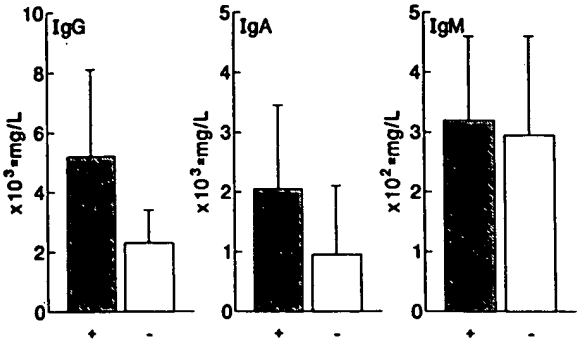


Figure 6. Plasma Immunoglobulin Levels

Plasma IgG, IgA, and IgM levels (means ± one S.D.) were compared between 15 *bcl*-2–Ig transgenic mice (lines M-23, M-30, M-43, M-57, and M-62, between 11 and 19 weeks of age; hatched bars) and normal littermates (open bars).

yet substantial, death rate during days 1–4. However, a subpopulation of approximately 10% of the transgenic splenocytes demonstrated extended survival in vitro for all lines assessed. Cultured splenocytes from transgenic mice displayed roughly a 500-fold survival advantage over matched normal littermates at day 14. Phase-contrast microscopy revealed only rare live cells from normal littermates, while numerous viable transgenic splenocytes were present as single cells and small aggregates (Figure 7B).

Flow cytometry was performed on the viable splenocytes in culture to determine their precise phenotype. The contour plots in Figure 7C demonstrate that of the surviving population, at day 17 in vitro more than 90% of cells expressed B220 and over 80% had detectable surface IgM/IgD. In contrast, less than 15% of cells reacted with anti-Thy-1, and reaction with anti-Mac-1 was negative. In addition, no AA4.1-positive cells were detected following culture, in contrast to the freshly obtained splenic B cells. Thus, after 2 weeks in culture nearly all surviving cells possess a mature B cell phenotype. Most of the B cells were small, but a subset of larger B cells was present also. Thymocytes from transgenics and normal littermates demonstrated the same rapid decline in viability in liquid culture (data not shown). Thus, the death-sparing effects conferred to B cells by the *bcl*-2–Ig transgene may be lineage specific. The cultured B cells from transgenic mice have remained viable for over 12 weeks in vitro. No spontaneous proliferation of these B cells in 5% fetal calf serum was noted when assessed by [^{3}H]thymidine incorporation. However, cultured B cells possessed the capacity to proliferate in response to appropriate stimuli, including lipopolysaccharide and supernatants of concanavalin A–activated spleen cells when assessed at day 21 (data not shown).

Discussion

The t(14;18) translocation that typifies human follicular B cell lymphoma juxtaposes the phenotypic landmark Ig gene with *bcl*-2, a recently discovered gene of unknown function (Tsujimoto et al., 1985; Bakhshi et al., 1985; Cleary and Sklar, 1985). The t(14;18) represents the best characterized example of a second generation of translocations that provide the opportunity to evaluate new putative proto-oncogenes. Whereas *bcl*-2 mRNA is normally expressed in pre-B cells, activated B cells, and activated T cells, the t(14;18) is found predominantly in mature B cell neoplasms of follicular center cells. Thus, the cell types that would be affected when a deregulated *bcl*-2 gene is inserted into the germ line of mice was uncertain.

bcl-2–Ig transgenes recreated the major event of the chromosomal breakpoint and resulted in the expansion of a follicular center B cell population. All transgenic lines developed hyperplastic splenic follicles that could fuse to form massive regions of splenic white pulp. The most advanced of these lesions are histologically similar to the follicular center cell lymphomas that develop spontaneously in susceptible mouse strains such as NFS/N, in which the early lesions are also confined to splenic white pulp (Pattengale and Taylor, 1983). Expression of the *bcl*-2–Ig transgene perturbs the lymphoid compartment, resulting in an increased percentage and absolute numbers of a B cell population bearing B220, Ia, IgM/IgD, and K. Provocatively, these cells display an unusually high AA4.1 positivity for what are otherwise mature B cells. Consistent with the notion that the severalfold-expanded population of IgM/IgD-bearing B cells is relatively immature, the serum IgM levels were normal. However, the presence of immunoblasts and plasma cells as well as the elevated serum IgG and IgA levels indicates that the transgene does not completely arrest maturation. Both forms of the minigene, bearing either the normal *bcl*-2 sequence (*bcl*-2–Ig NL) or the variations found in a human tumor line (*bcl*-2–Ig SM), have to date produced similar qualitative effects as transgenes. This finding places further emphasis upon gene deregulation and the elevated levels of *bcl*-2–Ig fusion RNAs found in follicular lymphomas as a primary mechanism of neoplasia.

Most remarkably, the *bcl*-2–Ig transgene has conferred a death-sparing capacity to a subset of splenocytes predominantly of mature B cell phenotype. These B cells have remained viable in simple liquid culture for over 12 weeks. Whether long-term autonomous cell lines will emerge in vitro is uncertain at this time. While these cells demonstrate little spontaneous proliferation, they are capable of responding to appropriate activation stimuli. These observations in transgenic mice are compatible with the effects that retroviral vector–introduced *bcl*-2 has shown on cell lines (Vaux et al., 1988). In that system, deregulated *bcl*-2 provides a short-term survival advantage to interleukin-3–dependent lines deprived of growth factor. While viable, such *bcl*-2–containing interleukin-3–dependent lines remain in G_0 and did not proliferate. Consistent with a limited transformation capacity for *bcl*-2, we have also noted that *bcl*-2 is not sufficient to confer tumorigenicity to lymphoblastoid cell lines, but it consistently improves their ability to be cloned in soft agar (Nunez et al., submitted). Moreover, deregulated *bcl*-2 did not produce classic transformed foci or morphologic changes in NIH 3T3 cells (Vaux et al., 1988; Nunez et al., sub-

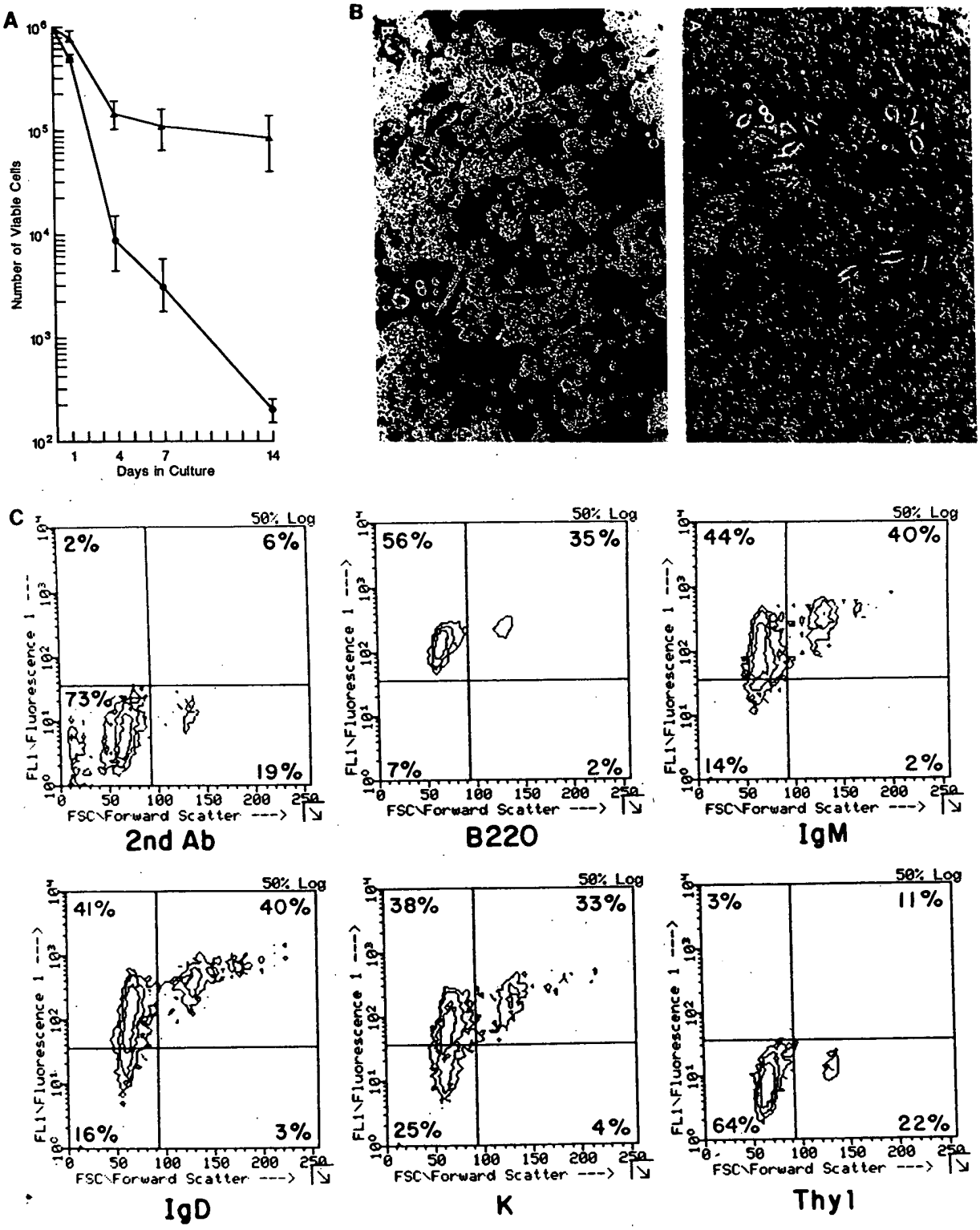


Figure 7. Survival of Splenocytes from Transgenic and Control Mice

(A) Viability plot of primary cultures of splenocytes from transgenics (triangles) and normal littermates (circles) as determined by trypan blue exclusion. Data points are the mean (± one S.D.) number of total viable cells remaining in the culture after initial plating at 1 × 10^6 per well. Plotted data represent cultures established from six transgenic animals (two each of the M-23, M-43, and M-62 lines) and three normal littermates.

(B) Phase-contrast photomicrographs of splenocyte cultures from a normal littermate (right) and an M-43 transgenic mouse at day 8 of liquid culture. Only rare viable, refractile cells are present in the normal culture, but numerous viable, refractile cells are present in the transgenic culture.

(C) Contour plots of viable splenocytes at day 17 of culture stained by indirect immunofluorescence and analyzed by flow cytometry. Contour plots were generated from analysis of 10,000 cells for each antibody. Contour levels were plotted with a threshold level of 10%. The horizontal cursor line was positioned on the basis of cells stained with second antibody alone. The vertical cursor line was arbitrarily placed to illustrate the variation in cell size.

mitted), although others noted that a subpopulation of *bcl*-2–transfected 3T3 cells could produce oligoclonal tumors in nude mice (Reed et al., 1988). The *bcl*-2–Ig transgene confers a survival advantage but not a strong proliferative signal, resulting in an apparently immortalized, expanded population of polyclonal B cells.

Human follicular lymphomas are monoclonal, reflecting the fact that the t(14;18) is acquired in a single somatic cell that clonally expands. In contrast, transgenic mice possess a deregulated *bcl*-2–Ig gene in their germ line. The effects of this transgene could potentially appear in all cells, and these mice develop a polyclonal expansion of mature B cells. Similarly, Eμ–*myc* transgenic mice develop a polyclonal hyperplasia of pre-B cells; however, they subsequently progress to monoclonal life-threatening tumors with a median age of onset at 9 weeks and illness or death at 12 weeks (Harris et al., 1988). This evolution of disease presumably results from the acquisition of additional genetic alterations. In contrast, *bcl*-2–Ig transgenic mice over 25 weeks of age demonstrate florid hyperplasia of splenic follicles, but have not progressed to monoclonality as yet. This difference must reflect the inherent properties of *myc* versus *bcl*-2. Perhaps *myc*-induced proliferation enhances the rate of acquiring additional genetic alterations (Langdon et al., 1986). Conversely, if the *bcl*-2–Ig transgene increases the survival but not the proliferation of B cells, it may result in a slower rate of acquiring complementing defects.

The results with the *bcl*-2–Ig transgenic mice are highly reminiscent of the disease from which *bcl*-2 was isolated. Human follicular lymphoma is often referred to as indolent lymphoma, in that relatively nonthreatening lymphadenopathy can exist for years and only progress to more aggressive disease with the acquisition of additional genetic changes (Fukuhara et al., 1979; Richardson et al., 1987). c-*myc* activation can be one of these events, but others are likely to predominate (Richardson et al., 1987; Gauwerky et al., 1988). *bcl*-2–Ig transgenic mice indicate that the pathologic consequence of the t(14;18) translocation is the survival and expansion of a mature B cell population. These mice provide evidence that the deregulation of *bcl*-2 is a primary oncogenic event. Moreover, *bcl*-2–Ig transgenic mice provide a prospective model to dissect the evolution of follicular B cell lymphomas.

Experimental Procedures

Construction of *bcl*-2–Ig Minigenes

The minigenes *bcl*-2–Ig NL (normal *bcl*-2 sequence) and *bcl*-2–Ig SM (encoding three amino acid differences found in line SU-DHL-6) (Figure 1) were generated by first constructing a specialized vector with a custom polylinker composed of tandem SalI, BamHI, PstI, EcoRI, HindIII, and SalI sites replacing the SphI–PvuII region of pGEM-1 (Promega, Madison, WI). Construction of *bcl*-2–Ig NL was initiated by digesting the vector with BamHI and EcoRI and inserting the 1.3 kb BamHI–EcoRI portion of *bcl*-2 cDNA no. 58 (Seto et al., 1988). This confirmed subclone was expanded, and a 4.6 kb BamHI–BamHI germ line genomic fragment containing the 5′ flank, promoters, exon I, intron I, and exon II up to the BamHI site was inserted. The correct orientation was selected, and an 11.6 kb EcoRI–EcoRI genomic fragment from the der (14) chromosome of SU-DHL-6 containing the *bcl*-2 MBR–J6–E_H–$C_{\gamma 1}$ fusion was inserted. The proper orientation was selected and expanded, and SalI was used to prepare the 17.5 kb fragment used for microinjection. *bcl*-2–Ig SM was generated in a parallel fashion except that the three amino acid differences encoded in the exon II portion of *bcl*-2 from SU-DHL-6 were included. This was accomplished by ligating a 3.6 kb BamHI–AccI 5′ germ line genomic fragment to the 1.3 kb AccI–PstI portion of cDNA no. 50 from SU-DHL-6 (Seto et al., 1988). This 5.3 kb ligated fragment was inserted into the BamHI and PstI sites of the polylinker, and the proper insert was confirmed. Digestion of this subclone with PstI and EcoRI enabled the insertion of a 1.0 kb PstI–EcoRI fragment of cDNA no. 58. Following expansion and confirmation, the 11.6 kb EcoRI–EcoRI *bcl*-2 MBR–J6–E_H–$C_{\gamma 1}$ fusion was inserted and the correct orientation selected.

Production of Transgenic Mice

Transgenic mice were generated as described by Hogan et al. (1986). Minigene constructs were resuspended in microinjection buffer (5 mM Tris [pH 7.4], 0.25 mM EDTA, and 5 mM NaCl) to a final concentration of 2–5 ng/μl. C57BL/6 × C3H/He F1 females were superovulated by sequential intraperitoneal injections of 5 U of pregnant mare serum (Sigma Chemical Co., St. Louis, MO) and 5 IU of human chorionic gonadotropin (Sigma) and mated with fertile F1 males. Single-cell embryos were harvested the following day using Brinster's BMOC-3 medium (GIBCO Laboratories, Grand Island, NY). Cumulus cells were removed by brief incubation in 300 μg/ml hyaluronidase. Pronuclei were microinjected with approximately 1–2 pl of minigene construct DNA, and two-cell embryos were reimplanted the following day into pseudopregnant ICR outbred female mice.

Primary Cultures

Single-cell suspensions were prepared from 8- to 9-week-old mice by mincing whole spleen or thymus and then pressing fragments between sterile frosted glass slides in Hanks' balanced salt solution. Organ capsules and connective tissue remnants were discarded. Cells were sedimented at 100 × g for 10 min, then resuspended in DMEM supplemented with 5% fetal calf serum and 100 U/ml penicillin–streptomycin. Viability at the time of initial plating was greater than 95% as determined by trypan blue exclusion. Cells were plated at a density of 1×10^6 mononuclear splenocytes or thymocytes in a volume of 0.5 ml in Costar 48-well plates. Cultures were maintained in 6% CO_2 at 37°C. Fresh medium was provided approximately every 2 weeks. Viability was determined by trypan blue exclusion and was expressed as total viable cells per well. Cell viability was confirmed by propidium iodide staining and analysis by flow cytometry. Cellular proliferation was assessed on days 1, 4, 7, and 14 by measuring incorporation of [^{3}H]thymidine (0.5 μCi per 5×10^6 cells plated in 96-well tissue culture plates [Falcon, Becton Dickinson Labware, Lincoln Park, NJ]).

Immunofluorescence Analysis of Single-Cell Suspensions

Preparation of single-cell suspensions for fluorescence analysis has previously been described (McKearn et al., 1984). In brief, 4×10^5 mononuclear cells derived from spleen, lymph node, or bone marrow were placed in individual wells of V-bottom 96-well microtiter plates. Cells were incubated for 30 min with optimal dilutions of the primary rat anti-mouse monoclonal antibodies. Cells were then washed twice and incubated with fluorescence-conjugated secondary antibody (see below) for 30 min. The cells were washed twice and resuspended in PBS that contained 0.1% bovine serum albumin and 2 μg/ml propidium iodide. Fluorescence was analyzed with a FACSCAN (Becton Dickinson, Sunnyvale, CA) on 1×10^4 single viable cells per sample.

Rat anti-mouse primary monoclonal antibodies have been previously described and included the following: 187.1, anti-mouse κ light chain (Yelton et al., 1981); JA12.5 anti-IgD (provided by the laboratory of J. M. Davie); 33-60, anti-IgM (Leptin et al., 1984); 14.8, anti-B lineage antigen B220 (Kincade et al., 1981); JIJ, anti-Thy-1.2 (Bruce et al., 1981); M5/114, anti-I-A and anti-I-E (Bhattacharya et al., 1981); AA4.1, anti-lymphohematopoietic antigen (McKearn et al., 1984); and GK1.5, anti-L3T4 (Dialynas et al., 1983). Secondary antibody consisted of a mixture of RG7/9, a mouse anti-rat κ light chain antibody, and RG7/11, a mouse anti-rat IgG2b antibody (Springer et al., 1982).

Tissue Sections

Tissues for light microscopy were fixed in 10% neutral buffered formalin and routinely processed for paraffin embedding. Sections 5 μm in thickness were stained with hematoxylin and eosin.

Tissue prepared for immunofluorescence staining was snap-frozen in O.C.T. compound (Miles Laboratories, Elkhart, IN) using liquid nitrogen. Frozen sections 5 μm in thickness were fixed for 2 min in 100% ethanol and air dried. Sections were blocked with 15% horse serum (Mazleton Research Products Inc., Denver, PA) in PBS for 30 min at room temperature, and incubated with optimal concentrations of the primary monoclonal antibodies, described above, for 30 min. Slides were washed three times in PBS and incubated with fluorescein-conjugated secondary antibody for 30 min. They were washed three times with PBS and mounted in glycerol containing p-phenylenediamine to retard fading of fluorescence (Johnson et al., 1982).

DNA Isolation and Analysis

Tail biopsies and mouse organs were digested overnight at 55°C in 2.5 ml of extraction buffer (50 mM Tris–HCl [pH 8.0], 100 mM EDTA, 100 mM NaCl, and 1% SDS) containing 1 mg of proteinase K (Beckman, Fullerton, CA). DNA was ethanol precipitated after four phenol–chloroform extractions. DNA pellets were washed twice with 80% ethanol, dried, and resuspended in 10 mM Tris (pH 7.4), 0.1 mM EDTA. Restriction endonuclease digestion by BamHI, PstI, or EcoRI, agarose gel electrophoresis, and Southern blotting were done using standard techniques (Maniatis et al., 1982). A 0.55 kb SacI–SacI human *bcl*-2 genomic fragment, including the 3′ portion of exon I, was used for screening for the integration and orientation of minigene constructs. In addition, a 1.0 kb XbaI–EcoRI mouse Ig J_H3–4 heavy chain genomic fragment was used to screen tissues for the presence of monoclonal immunoglobulin gene rearrangements. DNA probes were labeled with ^{32}P by random hexanucleotide priming (Feinberg and Vogelstein, 1983). Filters were hybridized overnight at 42°C in the presence of 10% dextran sulfate, 50% formamide, 4× SSC, 1× Denhardt's solution, and 10 μg/ml salmon sperm DNA. Blots were washed twice with 0.1% SDS, 2× SSC at room temperature, twice with 0.1% SDS, 0.1× SSC at 55°C, and exposed to Kodak XAR film at −70°C.

S1 Nuclease Protection Assay

Total cellular RNA was isolated from mouse organs including brain, heart, lung, thymus, liver, kidney, and spleen, essentially as described by Chirgwin et al. (1979). An [α-^{32}P]dCTP uniformly radiolabeled antisense single-stranded DNA probe possessing the *bcl*-2 exon III MBR was prepared by primer extension of an M13 clone. The DNA was digested with AvaI, and the single-stranded probe was prepared from an alkaline agarose gel. The probe (100,000 cpm) was hybridized to 20 μg of total cellular RNA at 53°C for 16 hr and then digested with 200 U of nuclease S1 for 1 hr at 37°C (Ley et al., 1982). RNA isolated from the human t(14;18) SU-DHL-6 cell line and the murine pre-B cell line 70Z/3 served as controls. Protected DNA fragments were size separated on a 6% sequencing gel, fixed, dried, and autoradiographed.

Radial Immunodiffusion

Serum was collected by puncture of the retro-orbital sinus with microcapillary tubes. Radial immunodiffusion kits for mouse IgG, IgA, and IgM were obtained from The Binding Site (Birmingham, England). The suggested serum dilutions and diffusion times were followed.

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